Gut, 1985, **26**, 1240–1245

Biliary excretion of radioactivity after intravenous administration of ^{3}H-1,25-dihydroxyvitamin D_3 in man

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SUMMARY Biliary radioactivity excretion was studied in 10 patients with postcholecystectomy T-tube drainage after intravenous administration of ^{3}H-1,25-dihydroxyvitamin D_3. The mean±SD radioactivity excreted in T-tube bile expressed as a percentage of the administered dose was 18·9±10·7% per 24 hours. After correction for incomplete bile collection the value obtained was 28·8±12·8%. The mean chloroform solubility of the biliary radioactivity increased from 17·0±8·4% to 69·4±15·1% after incubation with β-glucuronidase. High performance liquid chromatography of chloroform extracts of bile revealed that most of the eluted radioactivity was more polar than $1,25(OH)_2D_3$. The percentage radioactivity eluting as ^{3}H-$1,25(OH)_2D_3$ increased from approximately 2·4±1·9 to 16·2±8·0 after incubation with β-glucuronidase. We conclude that significant amounts of intravenously administered ^{3}H-$1,25(OH)_2D_3$ are excreted in bile, mostly as more polar metabolites. The increase in free ^{3}H-$1,25(OH)_2D_3$ after incubation with β-glucuronidase indicates that glucuronides of $1,25(OH)_2D_3$ are present in bile.

It is well established that vitamin D and its metabolites are excreted in bile, both in animals and in man.[1–9] The biliary excretion of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is of particular interest because this metabolite is believed to be the major active form of vitamin D. It is known to undergo side chain oxidation to calcitroic acid in the liver and intestine,[10 11] conversion to a 26,23-lactone in the intestine[12] and 24-hydroxylation in the kidney and intestine.[13] In addition, excretion of metabolites of $1,25(OH)_2D_3$ has been demonstrated in urine, bile, and faeces.[5] Weisner *et al*[9] reported a mean biliary excretion of 15·6% of an intravenous dose of ^{3}H-1,25-dihydroxyvitamin D_3 after six hours in five normal subjects, whereas in rats, dose dependent values of between 25 and 72%/24 hours were found.[6 8] These values are higher than those mostly reported for vitamin D and 25OHD[1 3 14–16] and suggest that biliary excretion may play a quantitatively important role in the metabolism of $1,25(OH)_2D_3$.

In this study we have measured biliary radioactivity excretion after intravenous administration of ^{3}H-$1,25(OH)_2D_3$ in 10 patients with T-tube drainage after cholecystectomy and have examined the nature of the radioactivity by high performance liquid chromatography.

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Received for publication 13 December 1984

Methods

PATIENTS

Ten patients (two men, eight women), aged between 23 and 73 years (mean 57 years), with postcholecystectomy T-tube drainage, were studied four to 13 days postoperatively (mean seven). No patient was receiving steroids, anticonvulsants, or vitamin D therapy. Liver function tests at the time of the study are shown in Table 1. All patients gave informed written consent and the study was approved by the ethical committee of St Thomas' Hospital.

PLASMA AND BILE BIOCHEMISTRY

Liver function tests were carried out on day one of the study using a Vickers M.300 Multichannel Analyser.

Plasma 25OHD concentrations were determined by a competitive protein binding assay[17] after extraction and chromatography using normal human serum as binding protein. The normal range, established in 57 subjects, 33 women and 24 men, aged 19–78 years (mean 42 years) was 12–80 nmol/l.

Plasma $1,25(OH)_2D_3$ concentrations were measured by a competitive protein binding assay after extraction and high performance liquid chromato-

Table 1 *Clinical details of patients studied*

Subject	*Sex*	*Age*	*Liver Function Tests*			*Plasma 25OHD (nmol/l)*	*Plasma 1,25 $(OH)_2D_3$ (pmol/l)*
			Pl AP (KA/dl)	*Pl Bi (μmol/l)*	*Pl ALT (IU/l)*		
Normal range			2–11	<19	<20	12–100	29–168
1	F	57	34	39	153	45	115
2	F	29	5	17	16	36	163
3	M	71	23	36	42	57	41
4	F	75	63	10	96	59	84
5	F	23	12	23	50	14	134
6	F	69	19	20	15	6	38
7	F	73	9	13	34	57	41
8	F	32	13	3	56	10	36
9	M	73	12	45	30	13	132
10	F	65	15	15	5	14	94

AP=alkaline phosphatase, Bi=bilirubin, ALT=alanine transaminase, 25OHD=25-hydroxyvitmin D, $1,25(OH)_2D_3$=1,25-dihydroxyvitamin D_3.

graphy.[18] The normal range, established in 28 subjects aged 20–30 years was 29–168 pmol/l.

CORRECTION FOR INCOMPLETE BILE COLLECTION

The bilirubin content of the bile was measured in order to calculate the percentage of total biliary secretions draining through the T-tube. For each collection period the biliary bilirubin concentration was estimated photometrically at λ530 using the diazo-reaction with p-iodoaniline by a modification of the method of Heirwegh *et al.*[19] Assuming a constant bilirubin production rate of 3·8±0·6 mg/kg/day (mean±SD) in man,[20] a correction factor was determined as follows:

$$\text{correction factor} = \frac{\text{calculated bilirubin excreted}}{\text{measured bilirubin excreted}}$$

The biliary radioactivity determined during each period was then multiplied by the correction factor. When bile volumes of less than 10 ml were obtained in one collection period, determination of the correction factor for this period was omitted and a correction factor derived from the other time periods was used.

MEASUREMENT OF BILIARY RADIOACTIVITY

Bile (0·5–1 ml) was freeze dried and bleached with 0·5 ml of 30% hydrogen peroxide and 0·5 ml of 0·5M HCl. The samples were then counted in 10 ml Micellar Scintillator NE260 (Nuclear Enterprises Ltd, Edinburgh, Scotland) using an LKB Rackbeta Liquid Scintillation Counter with an efficiency for tritium of 42%. Quench correction was applied using the channels ratio method. The mean recovery of tritium after addition of a known amount of ^{3}H-$1,25(OH)_2D_3$ to unlabelled bile was 92% (20 experiments).

EXPERIMENTAL PROTOCOL

1α,25-dihydroxy[23,24(n)-^{3}H] cholecalciferol, 192–216 mCI/mg (7·10–7·99 GBq/mg) was obtained from Amersham International plc, Amersham, Buckinghamshire, England. 1·7–5·6 μCi (62·9–207·2 Bq) containing 8·6–29·0 ng (mean 16·5) of $1,25(OH)_2D_3$ in 10% ethanol made up in 20 ml sodium chloride solution was injected intravenously over three to five minutes. The injected dose was calculated in individual patients after correction for losses of isotope occurring during the preparation of the injection.

BILE COLLECTION

Bile was collected in the dark on ice. Fractions 0–4 hours, 4–8 hours and 8–24 hours were collected and stored at −20°C until analysed.

MEASUREMENT OF CHLOROFORM SOLUBLE RADIOACTIVITY IN BILE

Aliquots of bile (0·5–1 ml) were added to 0·2 M acetate buffer (pH 5·05) in a 1:3 ratio; 4·7 fishman units of β-glucuronidase per dpm ^{3}H was added and the mixture was incubated for 24 hours at 37°C. This amount of enzyme per dpm tritium added gave optimum enzyme activity under these conditions.

Bile before and after β-glucuronidase (glucuronosohydrolase; EC 3.2.1.31) treatment was freeze dried and extracted twice with chloroform:methanol (2:1 vv)[21] (Bovine liver-glucuronidase Type B-10, was obtained from Sigma London Chemical Company Ltd, Poole, Dorset, England).

2000 dpm of ^{14}C-vitamin D_3 (specific activity 5·18 MBq/mg) (Amersham International plc) was added as an internal standard for the above extraction process. A mean recovery of 90% was obtained from six samples.

20 000 dpm of chromium-51-labelled sodium chromate in aqueous solution, (specific activity 9·25–18·5 GBq/mg chromium) (Amersham International plc) was added to bile samples in order to monitor carry-over of aqueous metabolites into the chloroform layer. The mean percentage ± SD ^{51}Cr carry-over was 2·5±2·1.

^{3}H and ^{14}C radioactivity was determined using a dual label program on an LKB Rackbeta Liquid Scintillation counter in 10 ml NE260 micellar scintillation fluid after bleaching with 0·5 ml 30% hydrogen peroxide and 0·5 ml 0·5 N hydrochloric acid. Counting efficiencies of 43% and 58% were achieved for ^{3}H and ^{14}C respectively. Chromium-51 radioactivity was measured by counting in a LKB Compugamma counter at ε 160, △ 16 with an efficiency of 7%.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF RADIOACTIVITY

Two hundred microlitres of ethanol containing 2 μg of $1,25(OH)_2D_3$ and 1 μg of $25OHD_3$ were added to dried 5 ml bile chloroform extracts which had been passed through a 0·45 μm filter (type HA, Millipore Corporation, Bedford, Massachusetts) and 100 μl was injected onto a 30 cm × 4 mm reverse phase MicroPak – MCH column (Varian Assoc Ltd, Walton-on-Thames, Surrey, England) on a Varian 5000 High Performance Liquid Chromatograph. A solvent system of methanol:water 80:20 (v/v) was used. The column was flushed with methanol after the appearance of the cold vitamin $25OHD_3$ marker. One hundred drop fractions were collected, and these were dried and counted for radioactivity. The mean recovery for standard bile extracts containing ^{3}H-$1,25(OH)_2D_3$ was 39·3% in 16 experiments. Each 100 drop fraction contained 2·8 ml, the flow rate being 2 ml/min. The elution time for $25OHD_3$ was 30·5 minutes and for $1,25(OH)_2D_3$, 11 minutes.

Results

The mean radioactivity excretion in T-tube bile per 24 hours was 18·9±10·7% (mean±SD) of the injected dose. After correction for incomplete collection of bile, this value increased to 28·8±12·8% (Table 2). Cumulative biliary radioactivity excretion in individual patients is shown in Fig. 1. There was no significant correlation between either the plasma 25OHD or $1,25(OH)_2D_3$ concentration and the percentage dose excreted in bile.

The mean percentage chloroform soluble radioactivity in 0–4, 4–8, and 8–24 hour bile samples was 18·8, 14·9 and 18·8% respectively. After incubation with β-glucuronidase, chloroform soluble radioactivity in these samples increased to 73·4, 65·6 and 65·6% respectively. High performance liquid chromatography of chloroform extracts before β-glucuronidase revealed that 2·5±4·7% of the eluted radioactivity was more polar than $1,25(OH)_2D_3$ (Fig. 2) and only small amounts of free ^{3}H-$1,25(OH)_2D_3$ were shown. After incubation with β-glucuronidase, however, 73·4±12·5% of eluted radioactivity was more polar than $1,25(OH)_2D_3$ and 16·2±8·0% comigrated with $1,25(OH)_2D_3$ (Fig. 3).

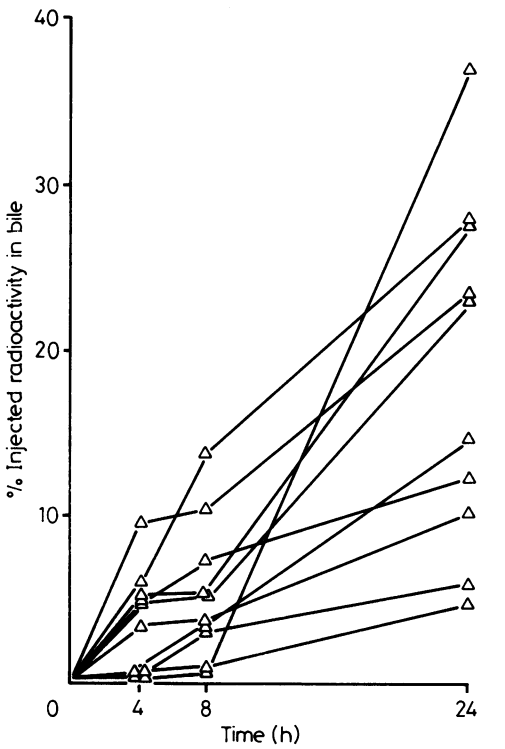

Fig. 1 *Cumulative biliary radioactivity excretion in the 10 patients, expressed as a percentage of the injected dose per 24 hours. Values are shown before correction for incomplete bile collection.*

Discussion

Our results show that approximately 18·9% of an intravenous dose of ^{3}H-$1,25(OH)_2D_3$ was excreted in T-tube bile per 24 hours in this group of postcholecystectomy patients. Excretion of radioactivity into T-tube bile after intravenous ^{3}H-$1,25(OH)_2D_3$ in man has not been previously reported; however, in the only other study carried out in man Weisner *et al*[9] found a mean six hour biliary radioactivity excretion of 15·6% in five

Table 2 *Biliary excretion of radioactivity after iv ^{3}H-1,25$(OH)_2D_3$ before and after correction for incomplete bile recovery*

Subject	Bile excreted in T-tube in 24h (ml) (vol)	Dose ^{3}H-1,25$(OH)_2D_3$ μCi	ng	Dose excreted in T-tube in 24h (%)	Dose excreted in 24h corrected with bilirubin correction factor (%)
1	146	3·8	17·6	4·7	23·7
2	206	4·3	22·4	23·3	31·9
3	619	5·6	29·2	23·5	23·5
4	236	4·0	20·8	15·1	31·3
5	459	1·7	8·6	37·1	57·7
6	316	2·5	11·7	12·5	22·5
7	440	2·9	12·9	28·0	36·1
8	334	2·7	14·4	28·0	NA
9	135	2·7	13·4	6·0	10·8
10	106	3·1	15·5	10·7	22·7

NA=not assessed.

normal subjects in whom bile was collected by duodenal intubation.

As bile collection from a T-tube is likely to be incomplete, we attempted to convert T-tube output to total bile output by comparison of the patient's calculated bilirubin production rate with reported normal values.[20] The correction factor thus derived, however, is likely to be subject to considerable error, because the normal bilirubin production rate was established in young subjects with normal liver function; moreover there may be considerable interindividual variation both in health and disease. Nevertheless, the mean corrected eight hour value of 12·6% is similar to the six hour values reported by

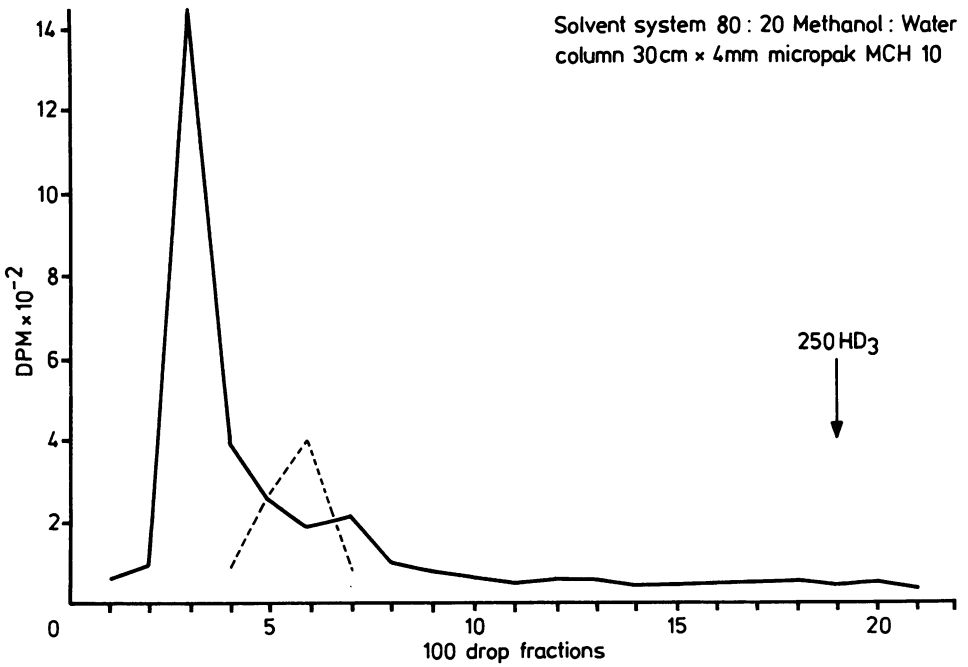

Fig. 2 *HPLC analysis of chloroform extracts of bile before β-glucuronidase treatment. The interrupted line shows the elution site of 1,25$(OH)_2D_3$.*

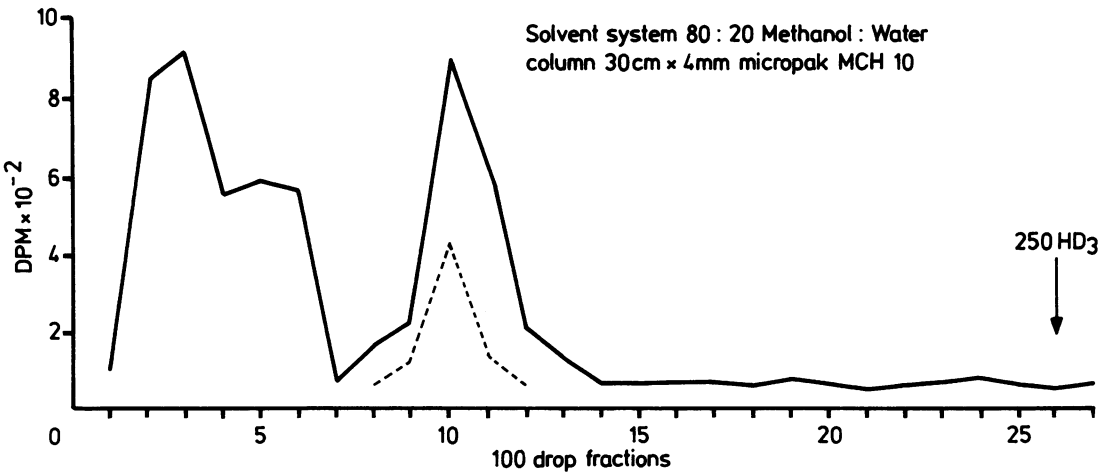

Fig. 3 *HPLC analysis of chloroform extracts of bile after β-glucuronidase treatment. The interrupted line shows the elution site of 1,25(OH)$_2$D$_3$.*

Weisner and coworkers.[9]

In agreement with the report by Kumar and colleagues[6] in rats, the majority of biliary radioactivity in this study was water soluble before incubation with β-glucuronidase. After the addition of enzyme, however, there was a large increase in chloroform soluble radioactivity, indicating the presence of glucuronides. The demonstration of greater amounts of free ^{3}H-1,25(OH)$_2$D$_3$ after than before incubation with β-glucuronidase provides the first evidence in man that glucuronides of 1,25(OH)$_2$D$_3$ may be present in bile; an increase in biliary radioactivity comigrating with 1,25(OH)$_2$D$_3$ after incubation with β-glucuronidase was reported by Kumar *et al* in rats receiving intravenous ^{3}H-1,25(OH)$_2$D$_3$ and recently Litwiller *et al*[22] have reported evidence for the presence of a monoglucuronide of 1,25(OH)$_2$D$_3$ in rat bile. Glucuronides of 25OHD have been identified in chick bile,[23] although their presence in human bile has not been definitely established.

Although the isotope given in this study was of high specific activity, the low plasma concentrations of 1,25(OH)$_2$D$_3$ normally circulating precluded the use of true tracer doses. Amounts of 1,25(OH)$_2$D$_3$ given to the subjects were approximately 10–30% of the endogenous plasma pool, as calculated by Gray *et al*;[5] these doses are approximately five to 20 times less than the smallest daily dose required to produce a demonstrable biological effect in normal adults.[24] Nevertheless, because of the doses of 1,25(OH)$_2$D$_3$ used in this study, the metabolism and disposition of the injected metabolite may have differed from that of endogenous metabolite. In addition, administration of the isotope as an intravenous bolus is unphysiological both in terms of rate of delivery to the circulation and the form in which it is administered. Because circulating 1,25(OH)$_2$D$_3$ is normally protein bound, hepatic uptake of injected metabolite might differ from that of endogenous metabolite.[25] Many of the patients studied had abnormal liver function; this may have affected the uptake and metabolism of injected ^{3}H-1,25(OH)$_2$D$_3$ by the liver, as well as possibly affecting biliary bilirubin excretion and hence affecting the correction factor for incomplete bile collection.

Our results, together with those reported by others in normal man[9] and animals[6 9] suggest that there is significant biliary excretion of the active form of vitamin D. The biliary metabolites have not been fully characterised, but glucuronides of 1,25(OH)$_2$D$_3$ appear to be present. The role of this enterohepatic circulation is at present unclear. Although intestinal reabsorption of biliary products of 1,25(OH)$_2$D$_3$ has been shown in rats[6] and man[9] the nature and biological activity, if any, of the reabsorbed metabolites is unknown. Vitamin D$_3$-3β-glucuronide and vitamin D$_2$-3β-glucuronide have been shown to have biological activity in the rat, although less than that of the parent vitamin.[26 27] The development of vitamin D deficiency and metabolic bone disease despite normal endogenous vitamin D synthesis in patients with intestinal malabsorption[28] and subjects receiving a high fibre diet[29] provides some indirect evidence to support the hypothesis that a quantitatively significant conservative enterohepatic circulation of vitamin D metabolites may exist. Alternatively, the entero-

hepatic circulation may play a purely excretory role. Finally, it is possible that the biliary products of $1,25(OH)_2D_3$ and other vitamin D metabolites may function within the intestinal lumen by assisting in the process of calcium and phosphate absorption from the intestine.

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